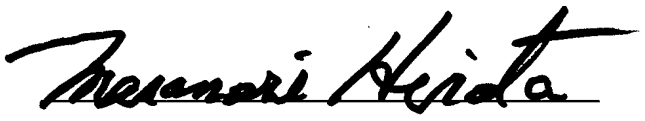
DECLARATION

I, Masanori Hirota of HIROTA PATENT OFFICE, residing at Wakabayashi Bldg., 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed at Tokyo, Japan, for a Japanese patent application under No. 2001-287698 on September 20, 2001 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION entitled: "ANIMAL MODEL WITH OVEREXPRESSION OF REGUCALCIN".

Signed this 16th day of January, 2006



Masanori Hirota

JAPAN PATENT OFFICE

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【Name of Document】 SPECIFICATION

【Title of the Invention】 ANIMAL MODEL WITH OVEREXPRESSION OF REGUCALCIN

【Scope of Claims】

【Claim 1】 A transgenic non-human animal to which a regucalcin gene is introduced and which overexpresses regucalcin.

【Claim 2】 The transgenic non-human animal according to claim 1, wherein straight chain DNA which is arranged in the order of cytomegalovirus-IE enhancer, chicken β-actin promoter, regucalcin gene, rabbit β-glovin poly A signal is introduced.

【Claim 3】 The transgenic non-human animal according to claim 1 or 2, wherein the regulcaltin gene is a gene that encodes protein consisting of amino acid sequence of Seq. ID No.2 of the sequence listing.

【Claim 4】 The transgenic non-human animal according to claim 3, wherein the gene encoding protein consisting of amino acid sequence of Seq. ID No.2 of the sequence listing is, a rat regucalcin gene consisting of DNA sequence of Seq. ID No.1 of the sequence listing.

【Claim 5】 The transgenic non-human animal according to any of claims 1 to 4, wherein the animal is homozygote.

【Claim 6】 The transgenic non-human animal according to any of claims 1 to 5, wherein the animal has an ability to suppress the weight gain.

【Claim 7】 The transgenic non-human animal according to any of claims 1 to 6, wherein the animal is susceptible to dysfunction of cerebrum.

【Claim 8】 The transgenic non-human animal according to any of claims 1 to 7, wherein the animal is susceptible to insulin independent diabetes.

[Claim 9] The transgenic non-human animal according to any of claims 1 to 8, wherein the animal is susceptible to renal hypertension.

[Claim 10] The transgenic non-human animal according to any of claims 1 to 9, wherein the animal is susceptible to impairment of tubular reabsorption.

[Claim 11] The transgenic non-human animal according to any of claims 1 to 10, wherein the non-human animal is a rat.

[Claim 12] A method for producing regucalcin, wherein the transgenic non-human animal according to any of claims 1 to 11 is used.

[Claim 13] A screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin, wherein the transgenic non-human animal according to any of claims 1 to 11, or tissues, organs or cells derived from the transgenic non-human animal and a test substance are used.

[Claim 14] The screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13, wherein the test substance is administered to the transgenic non-human animal, and the level of the weight gain of said transgenic non-human animal is measured and estimated.

[Claim 15] The screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is dysfunction of cerebrum.

[Claim 16] The screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is insulin independent diabetes.

[Claim 17] The screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is renal hypertension.

[Claim 18] The screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is impairment of tubular reabsorption.

[Claim 19] A preventive or therapeutic agent for diseases caused by the overexpression of regucalcin obtained by the screening method according to any of claims 13 to 18.

[Claim 20] A screening method of causative agents of diseases caused by the lowering of regucalcin expression wherein the transgenic non-human animal according to any of claims 1 to 11, or tissues, organs or cells derived from the transgenic non-human animal and a test substance are used.

[Claim 21] The screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20, wherein the test substance is administered to the transgenic non-human animal, and the level of the weight loss of the transgenic non-human animal is measured and estimated.

[Claim 22] The screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20 or 21, wherein the disease caused by the lowering of regucalcin expression is osteoporosis.

[Claim 23] The screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20 or 21, wherein the disease caused by the lowering of regucalcin expression is arteriosclerosis myocardial

infarction.

[Claim 24] The screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20 or 21, wherein the disease caused by the lowering of regucalcin expression is myocardial infarction.

[Claim 25] A causing substrate of disease caused by the lowering of regucalcin expression obtained by the screening method according to any of claims 20 to 24.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to regucalcin gene introduced transgenic non-human animal, more specifically to a transgenic non-human animal to which regucalcin gene is introduced and that has ability to suppress body weight gain, a method of producing regucalcin using said transgenic non-human animal, a screening method of preventive and theurapeutic agent of disease caused by the overexpression of regucalcin and a screening method of causative agent of disease caused by the lowering of regucalcin expression.

[0002]

[Prior Art]

Peptide hormone is bound to the receptor of the cell membrane and transmits the information into the cells. Ca^{2+} plays an important role in this mechanism. Many proteins that binds Ca^{2+} exist in the cells, an0d calmodulin plays an important role as a protein that amplify that action. It is clarified that Ca^{2+} is bound to said calmodulin, and activates various enzymes related to the regulation of cell functions (Science, 202, 19-27, 1984). Moreover, it is known that Ca^{2+} acts on protein kinase

C or other Ca^{2+} binding protein (including enzyme)(Science, 233, 305-312, 1986). Regucalcin is also a Ca^{2+} binding protein that was isolated from rat liver cytoplasm by the present inventor.

[0003]

Regucalcin is a Ca^{2+} binding protein whose molecular weight is 33388, wherein the Ca^{2+} binding constant shows $4.19 \times 10^{5}M^{-1}$, having 6 to 7 high-affinity Ca^{2+} binding sites, comprising 34% of α-helial structure, and is an acidic protein existing notably in liver, wherein the isoelectic point is pI5.20. Regucalcin is a specific protein that does not comprise a site EF hand structure (region) that is seen in calmodulin and many other Ca^{2+} binding proteins. For example, by binding Ca^{2+}, the calmodulin increases its α-helial content and its structure becomes robust, but regucalcin decreases the α-helial content. Moreover, on the other hand, it has been clarified that in the regulation of cell functions, regucalcin inhibits the enzyme activation caused by calmodulin and also inhibits the activation of protein kinase C. As described, there are many knowledges such as regucalcin functions as a regulatory protein for signaling (FEBS Lett, 327, 251-255, 1993).

[0004]

The regucalcin gene exists on chromosome X in rat (Xq 11.1-12), and is localized also in chromosome X in human. The regucalcin gene has been demonstrated in higher animals such as monkey, mouse, dog, bovine, rabbit, chicken and the like other than rat or human, but not yeast, and it is believed that it encodes highly differentiated protein. Regucalcin cDNA is cloned and all of its whole structure is determined (Japanese Laid-Open Patent Application No. 7-123985). As for the rat liver regucalcin cDNA, the base pair encoding whole amino acid is 0.897 kb, and it

translates 299 amino acids. Furthermore, the base sequence of regucalcin cDNA of the mouse liver or human liver is also determined, and by comparison with regucalcin cDNA of rat liver, they have 94% and approximately 89% of homology, respectively. The expression of regucalcin mRNA is observed in the liver of human, rat, mouse, bovine, chicken and the like, and in these livers, the presence of regucalcin protein has been also verified.

[0005]

The regucalcin is known to be a protein characterized as a regulatory protein of intracellular Ca^{2+} signaling having multifuncional role, and to be an important protein related to the regulation of cell functions (Life Sciences 66, 1769-1780, 2000, Biochemical and Biophysical Research Communications 276, 1-6, 2000). It has also been clarified by experiments to animals that the expression of regucalcin in liver or kidney of a living body is decreasing at the time of hepatopathy (Molecular and Cellular Biochemisty 131, 173-179, 1994) or nephropathy (Molecular and Cellular Biochemisty 151, 55-60, 1995), and the relation between regucalcin and cause of disease is suggested. There is a method to differentiate the serum of the patient having liver disease by measuring the serum of concentration of regucalcin, which exists specifically in kidney unlike the existing liver function markers such as GOT, GPT and the like, and as the regucalcin is increasing significantly in the serum of patient having liver disease, but on the other hand, almost no regucalcin is detected in the serum of a healthy person, therefore, said measurement is known to be useful for a differential diagnosis of serum of patient having liver disease (Japanese Laid-Open Patent Application No. 10-26623).

【0006】

【An Object to be Attained】

The regucalcin protein is a specific multifunctional protein specifically expressed in liver, which is expressed also with a low level, in kidney, heart, cerebrum (neuron), is engaged with the regulation of the cell function related to intracellular Ca^{2+} signaling. When its expression is lowered, it induces physiological abnormality. A functional analysis has been performed heretofore by using protein or anti-regucalcin monoclonal antibody isolated from rat liver, and the present inventor has clarified functional role of regucalcin in many living body regulation, beside a role as a regulatory factor of calcium signal mentioned above, which are regulation of cell nucleus function such as regulation of calcium transportation enzyme in the cells; a role as an activating factor of protease; regulation of cell nucleus function such as: regulation of calcium transport of cell nucleus; a role in the degradation of cell nucleus DNA; a role in cell nucleus function when regenerating liver; a role in the resorption of calcium in renal tubule and the like.

【0007】

The present inventor noted during the process of research for clarification of various functional roles of the regucalcin, that the regucalcin has specific effect different from many other Ca^{2+} binding proteins. The inventor perceived that the functional regulation of various cells to which calcium is related is formed on the balance of the expression level of regucalcin in vivo and the expression level of many other Ca^{2+} binding proteins such as calmoudulin and the like; and had decided to examine the change and effect occurred in the living body,

when the balance of the expression level of regucalcin and the expression level of many other Ca^{2+} binding proteins is disrupted. The object of the present invention is to provide animal model with overexpression of regucalcin, which is a tool to examine how the change and effect occur in a living body, when the balance between regucalcin and many other Ca^{2+} binding proteins is disrupted by overexpressing the regucalcin which is expressed primitively in the liver and the like of higher animals.

[0008]

[Means to Attain the Object]

The present inventor has made a keen effort to solve the above mentioned problems. The regucalcin cDNA was cloned from rat liver cDNA library, cDNA which encodes the full length of the regucalcin protein was isolated, ORF was cut from said rat regucalcin full length cDNA to be introduced into the expression vector (pCXN2). Said gene expression vector was microinjected into the male proneucleus of the rat fertilized egg, said fertilized egg was transplanted into the uterine tube of the foster parent rat to generate rats. DNA was extracted from the tissue of the generated rats, and rats in which regucalcin cDNA is integrated were determined by PCR. From 29 rats which have been generated, 5 homogeneous rats (4 males, 1 female) expressing regucalcin cDNA were constructed, and the present inventor has found that the weight gain of said transgenic rats were significantly suppressed. Thus, the present invention has been completed.

[0009]

In other words, the present invention relates to a transgenic non-human animal to which a regucalcin gene is introduced and which overexpresses regucalcin (claim 1); the transgenic

non-human animal according to claim 1, wherein straight chain DNA which is arranged in the order of cytomegalovirus-IE enhancer, chicken β-actin promoter, regucalcin gene, rabbit β-glovin poly A signal is introduced (claim 2); the transgenic non-human animal according to claim 1 or 2, wherein the regulcaltin gene is a gene that encodes protein consisting of amino acid sequence of Seq. ID No.2 of the sequence listing (claim 3); the transgenic non-human animal according to claim 3, wherein the gene encoding protein consisting of amino acid sequence of Seq. ID No.2 of the sequence listing is, a rat regucalcin gene consisting of DNA sequence of Seq. ID No.1 of the sequence listing (claim 4); the transgenic non-human animal according to any of claims 1 to 4, wherein the animal is homozygote (claim 5); the transgenic non-human animal according to any of claims 1 to 5, wherein the animal has an ability to suppress the weight gain (claim 6); the transgenic non-human animal according to any of claims 1 to 6, wherein the animal is susceptible to dysfunction of cerebrum (claim 7); the transgenic non-human animal according to any of claims 1 to 7, wherein the animal is susceptible to insulin independent diabetes (claim 8); the transgenic non-human animal according to any of claims 1 to 8, wherein the animal is susceptible to renal hypertension (claim 9); the transgenic non-human animal according to any of claims 1 to 9, wherein the animal is susceptible to impairment of tubular reabsorption (claim 10); the transgenic non-human animal according to any of claims 1 to 10, wherein the non-human animal is a rat (claim 11).

[0010]

Furthermore, the present invention relates to a method for producing regucalcin, wherein the transgenic non-human animal according to any of claims 1 to 11 is used (claim 12); a screening

method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin, wherein the transgenic non-human animal according to any of claims 1 to 11, or tissues, organs or cells derived from the transgenic non-human animal and a test substance are used (claim 13); the screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13, wherein the test substance is administered to the transgenic non-human animal, and the level of the weight gain of the transgenic non-human animal is measured and estimated (claim 14); the screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is dysfunction of cerebrum (claim 15); the screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is insulin independent diabetes (claim 16); the screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is renal hypertension (claim 17); the screening method of preventive and therapeutic agents for diseases caused by the overexpression of regucalcin according to claim 13 or 14, wherein the disease caused by the overexpression of regucalcin is impairment of tubular reabsorption (claim 18); and a preventive or therapeutic agent for diseases caused by the overexpression of regucalcin obtained by the screening method according to any of claims 13 to 18 (claim 19).

[0011]

Furthermore, the present invention relates to a screening method of causative agents of diseases caused by the lowering of regucalcin expression wherein the transgenic non-human animal according to any of claims 1 to 11, or tissues, organs or cells derived from the transgenic non-human animal and a test substance are used (claim 20); the screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20, wherein the test substance is administered to the transgenic non-human animal, and the level of the weight loss of the transgenic non-human animal is measured and estimated (claim 21); the screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20 or 21, wherein the disease caused by the lowering of regucalcin expression is arteriosclerosis myocardial infarction (claim 22); the screening method of causative agents of diseases caused by the lowering of regucalcin expression according to claim 20 or 21, wherein the disease caused by the lowering of regucalcin expression is myocardial infarction (claim 23); the screening method of causative agents of diseases caused by the lowering of regucalcin expression according to either claim of 20 to 23, wherein the disease caused by the lowering of regucalcin expression is myocardial infarction (claim 24); the causative agents of diseases caused by the lowering of regucalcin expression obtained by the screening method according to any of claims 20 to 24 (claim 25).

【0012】

【Mode for Carrying out the Invention】

As for the transgenic non-human animal of the present invention, there is no specific limitation as long as it is a non-human animal to which the regucalcin gene is introduced and

that overexpesses regucalcin. In the present invention, by the term "overexpress regucalcin", it means that a significantly larger amount of regucalcin is expressed compared to the expression level of regucalcin in wild-type non-human animal. Furthermore, as for non-human animal mentioned above, examples include rat, mouse, bovine, porcine, chicken, frog, human, dog, rabbit and the like, but rat is preferable among these examples. As for mouse that is frequently used as animal model, the organs are small and there is sometime a limit for analysis of pathology, but it is possible in rat to measure for example blood pressure and the like, and it is significantly useful as a means for animal experiments for clarification of pathology or gene therapy.

[0013]

As for a preferred embodiment for the transgenic non-human animal of the present invention, a transgenic non-human animal to which the straight chain DNA arranged in the order of cytomegalovirus-IE enhancer, chicken β-actin promotor, regucalcin gene, rabbit β-globin poly A signal is introduced can be exemplified. For example, when an expression vector (pCXN2) having marker gene, cytomegalovirus-IE enhancer, chicken β-actin promoter, cDNA insertion site, rabbit β-globin poly A signal and the like to which regucalcin full length cDNA is introduced is used, a transgenic non-human animal can be obtained effectively.

[0014]

Moreover, as a preferred embodiment for the transgenic non-human animal of the present invention, a transgenic non-human animal wherein the regucalcin gene is a gene that encodes protein consisting of the amino acid sequence of Seq. ID No.2 of the sequence listing, especially wherein the gene that encodes

protein consisting of amino acid sequence of Seq. ID No.2 of the sequence listing is a rat regulcacin gene consisting of DNA sequence of Seq. ID No.1 of the sequence listing can be exemplified, but the origin of the regucalcin gene is not limited to rat, and examples include mouse, bovine, porcine, chicken, frog, human, dog, rabbit and the like.

[0015]

Moreover, as for a preferred embodiment of the transgenic non-human animal of the present invention, a transgenic non-human animal that is homozygote can be exemplified. Said homogeneous that has homo on mutant chromosome can be generated by intercrossing non-human animals such as rats and the like having hetero on chromosome, and as the expression level of regucalcin is larger than heterozygote it is especially preferable for animal models used for experiments. Moreover, as for the transgenic non-human animal of the present invention, a transgenic non-human animal wherein the weight gain is significantly suppressed compared to wild-type non-human animal, in other words transgenic non-human animals having ability to suppress weight gain can be exemplified preferably. It was not possible at all to estimate that a transgenic non-human animal to which regucalcin gene is introduced and that overexpresses regucalcin has said ability to suppress weight gain, and from this new knowledge, it is suggested there is a possibility that regucalcin has a utility as preventive for obesity. From said new knowledge, the transgenic non-human animal of the present invention is a transgenic non-human animal wherein the regucalcin gene is introduced and that overexpresses regucalcin, and has ability to suppress weight gain.

[0016]

As for a preferred embodiment of the transgenic non-human animal of the present invention, a transgenic non-human animal expressing one or more of the following symptoms or diseases caused by overexpression of regucalcin: symptom of dysfunction of cerebrum, symptom of insulin independent diabetes, symptom of renal hypertension, symptom of impairment of tubular reabsorption and the like. It is believed that the dysfunction of cerebrum is developed by that the overexpressed regucalcin suppresses the activation of Ca-calmodulin dependent protein kinase that is necessary for the mechanism of cerebrum to maintain memory, and controls the neurotransmission in neuron. Therefore, the transgenic non-human animal of the present invention is useful as an experiment animal model for dysfunction of cerebrum such as memory (dementia such as Alzheimer disease and the like). Moreover, regucalcin expresses in kidney or liver, controls the intracellular signaling of the hormone, and due to the overexpression of regucalcin, the action expression of the hormone controlling the function of the liver and the kidney is impaired, and in the liver, as the function of insulin is suppressed, the insulin independent diabetes is induced. Furthermore, in the kidney, it is believed to induce renal hypertension related to the renin-angiotension system, and moreover the impairment of tubular reabsorption related to the metabolism of electrolyte. Therefore, the transgenic non-human animal of the present invention is useful as an experiment animal model of insulin independent diabetes, renal hypertension, impairment of tubular reabsorption and the like.

【0017】

As for the method for establishing animal models such as model rats and the like, having ability to suppress weight gain,

of the present invention, a method using the method for preparing transgenic animals which is already known (for example, Proc. Natl. Acad. Sci. USA 77:7380-7384, 1980) can be exemplified. For example, as for a method for generating a regucalcin (RC) transgenic rat, a method as follows or the like can be exemplified: the regucalcin cDNA is cloned from rat liver cDNA library, cDNA which encodes the full length of regucalcin protein is isolated, open reading frame (ORF) is cut from said rat regucalcin full length DNA to be introduced into the expression vector, a straight chain DNA fragment comprising a transgene prepared by linealizing said gene expression vector is microinjected into the male pronucleus of the rat fertilized egg, and said fertilized egg or the embryo of 2 cells period is transplanted to the uterine tube of the foster parent rat to generate rats, and DNA is extracted from the tissue of the generated rats to determine by PCR that regucalcin cDNA is integrated.

[0018]

As for the method for preparing regucalcin of the present invention, there is no specific limitation as long as it is a method using a transgenic non-human animal of the present invention, preferably a homozygous transgenic non-human animal. For example by removing the liver from a homozygous regucalcin transgenic rat, the regucalcin can be isolated and purified from the homogenate according to a method described previously (Chem. Pharm. Bull. 26, 1915-1918, 1978). Moreover, for the purpose of yield increase of the regucalcin, calcium, calcitonin, insulin, estrogen and the like can be administered to transgenic non-human animals.

[0019]

As for the screening method of preventive and therapeutic

agents for diseases caused by overexpression of regucalcin of the present invention, there is no specific limitation as long as it is a method that uses a transgenic non-human animal of the present invention or tissues, organs or cells derived from the transgenic non-human animal and test substances, and as for diseases caused by overexpression of regucalcin, examples include dysfuntion of cerebrum, insulin independent diabetes, renal hypertension, impairment of tubular reabsorption and the like. As for the method of using transgenic non-human animal and test substance as described above, examples include: a method of administering directly test substances to transgenic non-human animals, measuring and estimating the level of weight gain of transgenic non-human animals, and the level of diseases caused by the overexpression of regucalcin; a method of measuring and estimating the level of suppression of regucalcin expression in tissues, organs or cells obtained from the transgenic non-human animal after test substances were administered; and a method of estimating the morphological change in tissues or organs by immunostaining with monoclonal antibody or by using electron microscope, and the like. As for a method using tissues, organs or cells derived from the transgenic non-human animal and test substances, examples include: a method of culturing tissues, organs or cells derived from transgenic non-human animals under the presence of test substances, then measuring and estimating the level of suppression of regucalcin expression in said tissues, organs or cells; and a method of estimating the morphological change in tissues or organs by immunostaining with monoclonal antibody or by using electron microscope, and the like.

[0020]

As for tissues or organs described above, liver, renal tubule, heart, cerebrum and the like can be exemplified concretely, and as for cells, hepatocytes or neurons constituting these tissues or organs can be exemplified concretely. Moreover, when screening these, it is preferable to compare and estimate with wild-type non-human animal, especially with litter wild-type non-human animal, as it enables to perform accurate comparative experiment at individual level. By this way, according to the screen method of the present invention described above, it is possible to screen preventive or therapeutic agents for diseases caused by the overexpression of regucalcin such as dysfunction of cerebrum, insulin independent diabetes, renal hypertension, impairment of tubular reabsorption and the like, and the preventive or therapeutic agents obtained by said screening method are included in the scope of the present invention.

[0021]

As for the screening method of causative agents of diseases caused by the lowering of regucalcin expression of the present invention, there is no specific limitation as long as it is a method using the transgenic non-human animal of the present invention, or tissues, organs or cells derived from the transgenic non-human animal and a test substance, and as for diseases caused by the lowering of regucalcin expression, arteriosclerosis, myocardial infarction and the like can be exemplified. As for the method using transgenic non-human animals described above and a test substance, examples include: a method of administering directly test substances to transgenic non-human animals, then measuring and estimating the level of weight loss of transgenic non-human animals and the level of disease caused by the lowering of regucalcin expression; a method

of measuring and estimating the level of increase of regucalcin expression in tissues, organs or cells obtained from the transgenic non-human animal after being administered test substances; and a method of estimating the morphological change in tissues or organ by immunostaining with monoclonal antibody or by using electron microscope, and the like. As for a method using tissues, organs or cells derived from the transgenic non-human animal and a test substance, examples include: a method comprising the step of culturing tissues, organs or cells derived from transgenic non-human animals under the presence of a test substance, then measuring and estimating the level of increase of regucalcin expression in said tissues, organs and cells; and a method of estimating the morphological change in tissues or organs by immunostaining with monoclonal antibody or by using electron microscope, and the like.

[0022]

As for tissues or organs described above, liver, renal tubule, heart, cerebrum and the like can be exemplified concretely, and as for cells, hepatocytes or neurons constituting these tissues or organs can be exemplified concretely. Moreover, when screening these, it is preferable to compare and estimate with wild-type non-human animal, especially with litter wild-type non-human animal, as it enables to perform accurate comparative experiment at individual level. By this way, according to the screening method of the present invention described above, it is possible to screen preventive or therapeutic agents for diseases caused by the lowering of regucalcin expression such as arteriosclerosis, myocardial infraction and the like. The causative agents of disease caused by the lowering of regucalcin expression obtained by said

screening method is useful to further clarify the action and role of regucalcin in the living body. Furthermore, by screening substances inhibiting its action, such as substances binding to these causative agents, it is also useful as there is a possibility to develop preventive or therapeutic agents for diseases caused by the lowering of regucalcin expression, and therefore, said causative agents are also included in the scope of the present invention.

[0023]

[Example]

The present invention will be explained in detail with examples in the following, but the technical scope of the present invention is not limited to these examples.

Example 1 (Preparation of rat RC cDNA)

(Preparation of RNA)

Liver was extracted from a male Wistar rat (3 weeks old), and homogenized with guanidine-isothiocyanate solution (4M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and 2 M sodium acetate). This mixture was extracted with phenol-chloroform-isoamyl alcohol mixed solution, and centrifuged at 4° C, 10,000 × g for 20 minutes. Isopropanol was added to the aqueous layer, left at -20° C to precipitate RNA. The precipitate was recovered, and dissolved in 0.5% sodium dodecyl sulfate treated with diethylpyrocarbonate. The resultant was put in an olygo (dT)cellulose column to purify poly (A) + RNA.

[0024]

(Preparation of cDNA library)

50 units of Moloney-Murine Leukemia virus reverse transcriptase and oligo (dT) 18 primer linker were added to

purified poly (A) + RNA (5 μg), to synthethize a single-strand cDNA. E. coli RNase H and DNA polymerase I were added to said synthethized single-strand cDNA to synthethize double-strand cDNA. EcoRI adapeter was added to this, and connected with phage expression vector (λZAPII) which was previously digested with XhoI and EcoRI. Further, by using packaging extract, the phage of cDNA library packaged to phage was prepared.

[0025]

(Selection of RC cDNA clone)

Approximately 1 x 10^6 of phage of rat liver cDNA library was mixed with E. Coli, and planted in 20 agar plates. After incubation at 42° C for 3 hours and half, the nitrocellulose membrane treated with 10 mM isopropyl thio β-D-galactoside was placed on the plate, and was incubated at 37° C for 3 hours and half. The nitrocellulose membrane was blocked, and then incubated with anti RC rabbit serum (× 200) at room temperature for 2 hours. The membrane was washed and then alkaline phosphatase conjugated anti-rabbit IgG antibody was added for incubation. The resultant was submerged in a coloring solution (0.35 mM nitroblue tetrazolium, 0.4 mM 5-bromo-4-chloro-3-Indolyl Phosphate) for coloring, and RC cDNA positive plaque was identified.

[0026]

(Subcloning to plasmid vector)

Phage vector λZAPII includes the base sequence of pBluescript being the plasmid vector in its sequence. The RC cDNA fragment cloned in λZAPII was inserted into this pBluescript. Moreover, at the both ends of pBluescript, an initiation point and termination point of replication of helper phage exist. The phage was isolated from the plaque determined here, was infected

with E. Coli SURE and R408 helper phage, pBluescript including RC cDNA fragment was synthethized inside E. Coli, and was released outside E. Coli in form of helper phage. This phage solution was further infected with E. Coli SURE, and replicated in the fungus as a plasmid having RC cDNA fragment. This E. Coli was implanted to LB plate comprising 50μg/ml of Ampicillin and ampicillin-resistant colony was selected.

【0027】

(Determination of the base sequence of cDNA insert)

All of the base sequence of cDNA insert was determined by using Sequenase system (US Biochemical). In other words, plasmid DNA was truncated with EcoRI, the fragment was alkalinized degeneratively and added with a primer and annealed. The resultant was divided in 4 after added with 35S dCTP, 0.1 M DTT, enzyme solution for sequenase solution. ddATP, ddGTP, ddTTP, ddCTP were added to each, and incubated at 37°C for 5 minutes. The resultants were isolated by electrophoresis on acrylamide gel, the autoradiography to read the base sequence was performed. All of the base sequence of regucalcin cDNA is shown in Seq. ID No.1. Further, the amino sequence obtained is also shown in Seq. ID No.2. The molecular weight of regucalcin calculated from this, was 33,388. This value was the same with the one calculated with regucalcin purified by the electrophoresis on SDS polyacrylamide.

【0028】

Example 2 【Generation of transgenic rat】

(Construction of transgene)

From the plasmid containing rat regucalcin full length cDNA obtained in Example 1, RC-900 (glycerol stock; RC-F), vector pBluescript SK (-), DNA fragment containing all ORF was resected

with PstI (Figure 1A). Said PstI fragment resected was integrated into PstI site of pBluescript II KS (+) (Figure 1B). Then, EcoRI fragment obtained by resecting with EcoRI (Figure 2A) was introduced into EcoRI site of the expression vector pCXN2 (Clontech) (Gene 108, 193-199, 1991) (Figure 2B) to prepare rat regucalcin expression vector RC/pCXN2. Said RC/pCXN2 was resected with SalI, SfiI and MluI to obtain a linealized 3.6 kbp fragment (Figure 3).

[0029]

(Preparation of transgenic rats)

The microinjection to rat prenuclear fertilized egg of 3.6 kbp DNA fragment solution linealized as described above was conducted as follows. A 4-weeks-old Sprague-Dawley (SD) female rat was raised in a light-dark cycle for 12 hours (light hours 4:00 - 16:00), at a temperature approximately of 23°C, a humidity approximately of 55%, and the female estrous cycle was observed by vaginal smear method, and the hormone treatment day was selected. 150 IU/kg of a pregnant horse serum gonadotrope (Nippon Zenyaku "PMS Zenyaku") was administered intraperitoneally to a female rat to perform superovulation. 48 hours later, 150 IU/kg of human placental gonadotrope (Sankyo Yell "PUBEROGEN") was administered intraperitoneally, and intercrossed with a male by cohabiting. 32 hours after the administration of human placental gonadotrope, prenuclear fertilized egg was collected by tubal superfusion.

[0030]

The 3.6 kbp DNA fragment solution (concentration of 5 ng/μl) were microinjected to a male pronucleus of fertilized egg of Wistar rats thus generated. The egg to which DNA fragment is injected was cultured overnight by using m-KRB (m-Krebs Ringer

buffer solution) medium in CO_2 incubator. The development get through 2-cell phase on the next day, 2-cell phase embryo with no abnormality were transplanted into uterine tubes of 9 foster parents (pseudopregnant female rat intercrossed with male wherein deferent canal is ligated) at a rate of 20 - 30 per rat, and 29 rats were generated. DNA was collected from tails of 27 rats that were generated and were alive until being 4-weeks old. The DNA collected were determined by PCR with the use of primer huRC-1; GGAGGCTATGTTGCCACCATTGGA (Seq. ID. No.3); primer huRC-2; CCCTCCAAAGCAGCATGAAGTTG (Seq. ID. No.4). As a result, the presence of the transgene was identified in a total of 5 rats (4 males and 1 female). Among these, 5 rats transmitted the transgene to the next generation.

[0031]

Example 3 [Ability to suppress weight gain]

Among strains of transgenic rats (heterozygote) obtained in Example 2, the strains which the amount of regucalcin expressed in tail tissue was largest were intercrossed to generate transgenic rat (homozygote). Furthermore, it was identified to be homozygote by determining the integration of transgene to genomic DNA extracted from rat tail tissue by PCR, and the integrated amount detected was more than 2 times of the cDNA amount of heterozygote. The ability of suppressing weight gain was examined by using said homozygote transgenic mouse. The average level of body weight of 3-4 weeks old wild-type SD rats and transgenic rats (homozygote) for 8 rats each are shown in Table 1. Student's t test, $P<0.01$, it is shown by mean value ± S.E.M. so a significant difference is considered. It was verified that the weight gain is suppressed by the overexpression of regucalcin gene.

【0032】

【Table 1】

	body weight (g)
wild-type	88.5±3.8
transgenic	69.5±2.4*

【0033】

【Effect of the Invention】

The regucalcin transgenic non-human animal of the present invention, especially the regucalcin transgenic rat, is useful for animal model for evaluating pathology such as adult diseases, lifestyle-related disease, geriatric disease and the like related to Ca^{2+} signaling, such as impairment of liver, renal disorder, diabetes, myocardial infarction, hypertension, Alzheimer's disease and the like. Moreover, the regucalcin is regulating cell function related to intracellular Ca^{2+} signaling, and as the regucalcin transgenic non-human animal of the present invention overexpresses said regucalcin, the animal can be a useful means as animal model for developing therapeutic drugs for gene therapy for repair/improvement of diseases specific to organs (cancer of liver, myocardial infarction, cerebrum dementia).

【0034】

【SEQUENCE LISTING】

SEQUENCE LISTING

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<120> Regucalcin gene-transferred non-human animals

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Gly Glu Ser Pro Val Trp Glu Glu Ala Ser Lys Cys Leu Leu Phe Val
20 25 30

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Asp Ile Pro Ser Lys Thr Val Cys Arg Trp Asp Ser Ile Ser Asn Arg
35 40 45

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Val Gln Arg Val Gly Val Asp Ala Pro Val Ser Ser Val Ala Leu Arg
50 55 60

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Gln Ser Gly Gly Tyr Val Ala Thr Ile Gly Thr Lys Phe Cys Ala Leu
65 70 75 80

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Asn Trp Glu Asp Gln Ser Val Phe Ile Leu Ala Met Val Asp Glu Asp
85 90 95

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Lys Lys Asn Asn Arg Phe Asn Asp Gly Lys Val Asp Pro Ala Gly Arg
100 105 110

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Tyr Phe Ala Gly Thr Met Ala Glu Glu Thr Ala Pro Ala Val Leu Glu
115 120 125

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130 135 140

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Lys Tyr Phe Asn Gln Val Asp Ile Ser Asn Gly Leu Asp Trp Ser Leu
145 150 155 160

gac cat aaa atc ttc tac tac att gac agc ctg tcc tac act gtg gat 528

Asp His Lys Ile Phe Tyr Tyr Ile Asp Ser Leu Ser Tyr Thr Val Asp
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gcc ttt gac tat gac ctg cca aca gga cag att tcc aac cgc agg act 576
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180 185 190

gtt tac aag atg gaa aaa gat gaa caa atc cca gat gga atg tgc att 624
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Lys Lys Asn Asn Arg Phe Asn Asp Gly Lys Val Asp Pro Ala Gly Arg
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Lys Tyr Phe Asn Gln Val Asp Ile Ser Asn Gly Leu Asp Trp Ser Leu
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Asp His Lys Ile Phe Tyr Tyr Ile Asp Ser Leu Ser Tyr Thr Val Asp
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[Brief Explanation of Drawings]

[Fig. 1]

Figure 1 is a figure that shows the process of cutting the ORF part from the full length cDNA of rat regucalcin, during the construction of the expression vector to generate the transgenic rat of the present invention.

[Fig. 2]

Figure 2 is a figure that shows the process of introducing the ORF part of the full length cDNA of rat regucalcin into the expression vector pCXN2, during the construction of the expression vector to generate the transgenic rat of the present invention.

[Fig. 3]

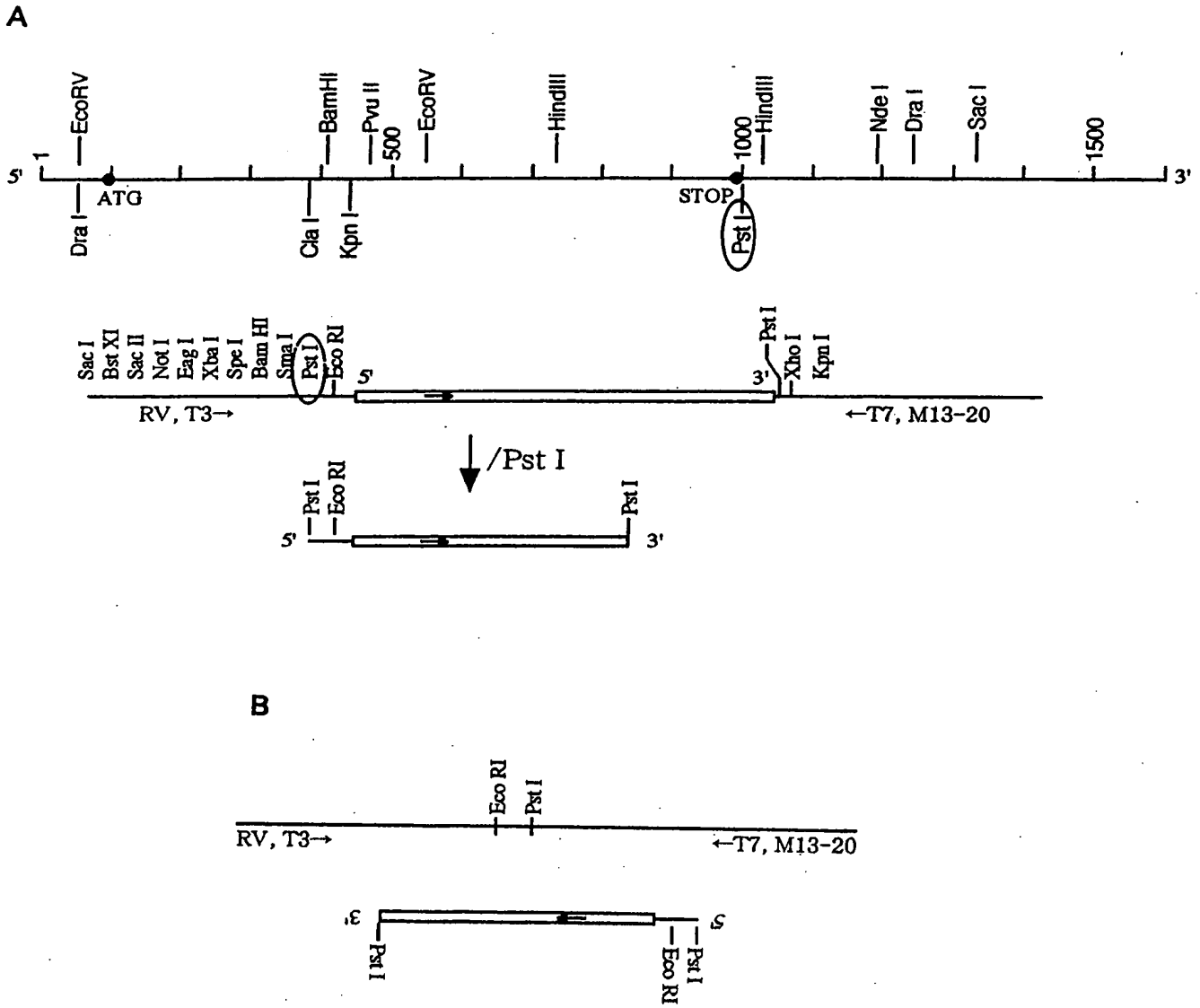
Figure 3 is a figure that shows the process of preparing the transgene fragment that has been linealized to generate the transgenic rat of the present invention.

[Fig. 4]

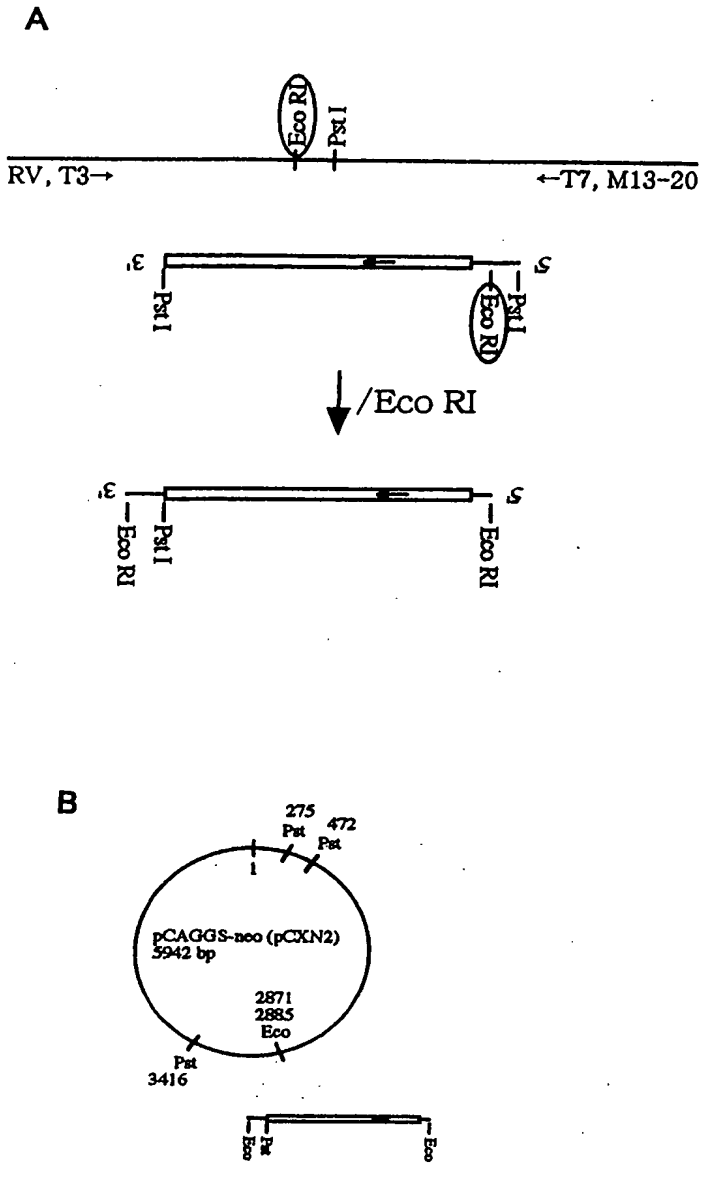
Figure 4 is a figure that shows the position of the primer during determination by PCR of the regucalcin gene in the transgenic rat of the present invention.

[Name of Document] DRAWINGS

[Fig. 1]



[Fig. 2]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5'
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Pst I
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3'
5'
Eco RI
Pst I
Eco RI
B
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Pst
472
Pst
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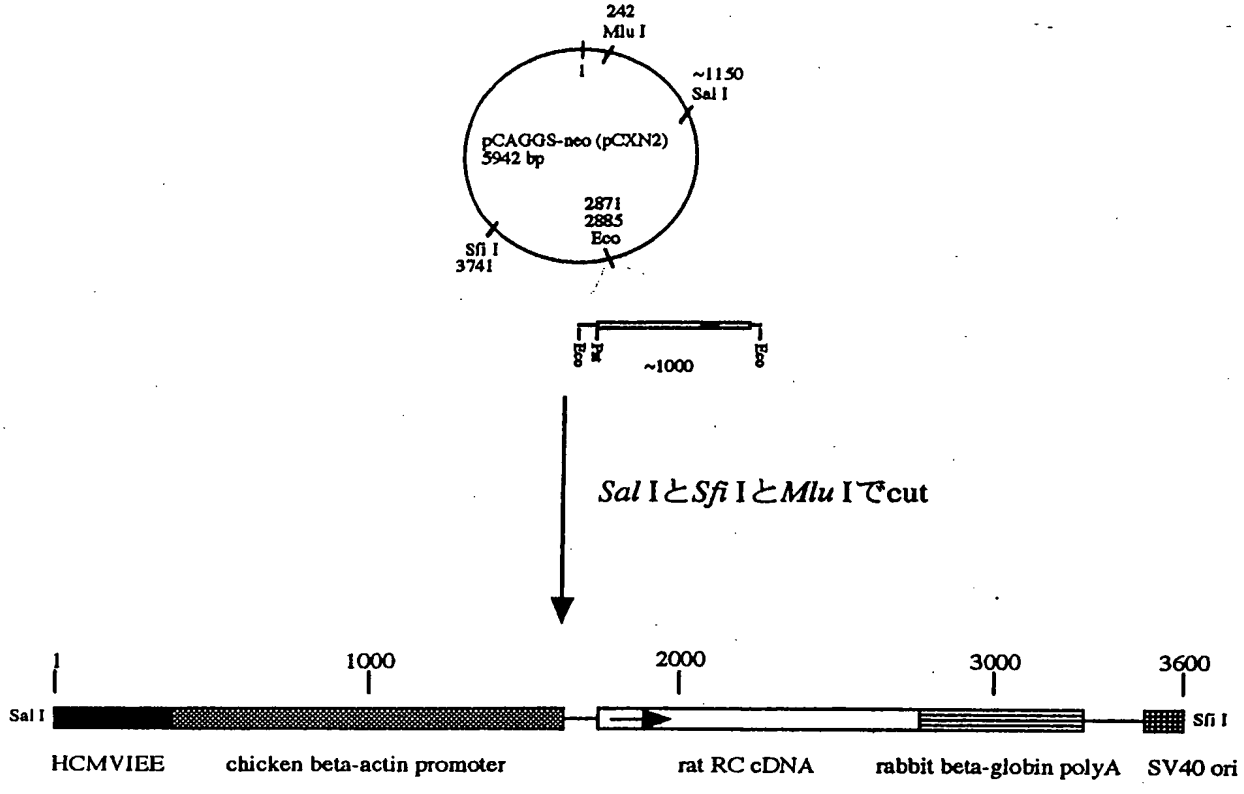
Eco

[Fig. 3]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Eco
Mlu
Eco
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Sal I と Sfi I と Mlu I で cut
1
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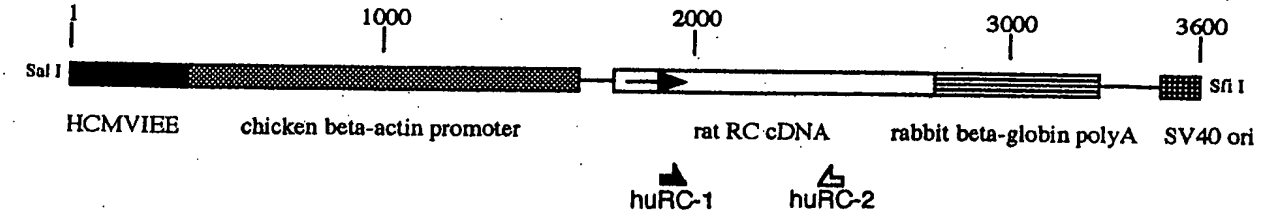
[Fig. 4.]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huRC-1
huRC-2

【Name of Document】 ABSTRACT

【Abstract】

【The Object】 The present invention provides an animal model with overexpression of regucalcin that overexpresses regucalcin, which is inherently expressed in the liver and the like of the higher animal, showing bone pathology typified by osteoporosisThe object of the present invention is to provide.

【Solving Means】 Regucalcin cDNA is cloned from rat liver cDNA library, and cDNA encoding the full-length of regucalcin protein is isolated. ORF is cut from said rat regucalcin full length cDNA and introduced into an expression vector (pCXN2). Said gene expression vector is microinjected to male pronucleus of the fertilized egg of rat. Said fertilized egg is tranplanted into the uterine tube of a host rat to generate rats. Thus, homozygous rats are constructed from said generated rats. Said transgenic rats show remarkable bone pathology, morphologically as well as biochemically, and the body weight gain is suppressed significantly.